

# CCXLVI. VITAMIN C AND THE SUPRARENAL CORTEX.

## I. ANTISCORBUTIC ACTIVITY OF OX SUPRARENAL.

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*(Received October 31st, 1932.)*

THE chemical study of vitamin C has been notably advanced by the discovery that hexuronic acid possesses antiscorbutic properties. It will be recalled that when he first isolated hexuronic acid, Szent-Györgyi [1928] concluded that it was identical with the reducing substance which is found in active vitamin C concentrates. However, observations by Zilva seemed to show that the reducing substance in question bore no relationship to vitamin C, and hence the matter was dropped for some years. In the meantime a series of detailed investigations by Tillmans and his collaborators [1930; 1932] led to the conclusion that antiscorbutic activity went strictly parallel with reducing capacity and it was argued that the reducing substance was "the carrier of vitamin C activity." Recently the whole question has been re-opened by Svirbely and Szent-Györgyi [1932, 1, 2], who reported that a specimen of hexuronic acid (derived from ox suprarenals) in doses of 1 mg. per day would protect guinea-pigs from scurvy over a 90-day test period. Independently King and Waugh [1932; see also Waugh and King, 1932] have found that a crystalline preparation of vitamin C which they isolated from orange juice possesses the chemical and physical properties of hexuronic acid. The conclusion which Szent-Györgyi draws from his test that "vitamin C is a single substance identical with hexuronic acid" is disputed by Zilva [1932, 1], although he admits [1932, 2] that specimens of hexuronic acid may contain some vitamin C. Zilva [1932, 3] opposes also the conclusions of Tillmans.

In a previous communication from this laboratory [Harris, Mills and Innes, 1932] confirmation was obtained, by means of striking curative tests and also by the microscopic tooth-structure method, of the conclusion which Svirbely and Szent-Györgyi had reached from a preventive test, that hexuronic acid possesses antiscorbutic potency; and its degree of activity was also determined quantitatively. The question was discussed whether the antiscorbutic action of hexuronic acid was an inherent property of the pure substance itself

or whether possibly it was due to traces of some associated impurity. It was pointed out that the theory that the vitamin and hexuronic acid are one and the same substance demands that the raw suprarenal cortex should possess an exceptionally high antiscorbutic activity, since this organ is known to be an even richer source of hexuronic acid than is orange juice itself. This we have found to be the case, the degree of activity of the cortex being in fact proportional to its high content of hexuronic acid<sup>1</sup>. A description of these experiments forms the subject of the present paper<sup>2</sup>.

### EXPERIMENTAL.

Supplies of fresh ox suprarenal were obtained daily or every second day from local slaughter-houses and used with as little delay as possible, being kept in the mean time in cold store in an atmosphere of CO<sub>2</sub>. On removal from cold store, the medulla was dissected away from the cortex, and the latter, after being passed through a fine wire sieve, was suspended in water and immediately administered by means of a dropper to the guinea-pigs used as experimental animals. One object of feeding the cortex only and removing the medulla was to avoid any possibility of adrenaline poisoning. It was also desirable to administer the vitamin in as concentrated a form as possible (there is no evidence of any being present in the medulla) since earlier experience in this laboratory has shown that guinea-pigs fail to thrive if given too large amounts of animal tissue.

The antiscorbutic activity of the suprarenal cortex was determined (1) by curative tests, and (2) by the microscopic tooth structure method.

#### 1. *Curative tests.*

This method has been used successfully in previous work published from this laboratory [*e.g.* Harris, Mills and Innes, 1932; Mills, 1932]. Matched guinea-pigs, males only, weighing about 250 g. are first placed for a preliminary period of about 10 days on the scorbutic basal diet:

Bran	...	...	...	80 parts by weight
Oats	...	...	...	720     "
Egg-yolk	...	...	...	40     "
Salts	...	...	...	8.4     "
Cod-liver oil	...	...	...	1 %

supplemented by 15 g. per day of cabbage, and next on the basal diet alone. Within a further period of 2 to 3 weeks animals are chosen for test which are showing early symptoms of scurvy, have begun to lose weight evenly and have dropped sharply 10 to 20 g. from their maximum weights (in the course of about 3 days) (see figure). Any animals whose weight curves have been lacking in smoothness or who have shown a departure from the average normal response are discarded, as also are those giving indications of scurvy complicated by infection (a not infrequent occurrence).

<sup>1</sup> Preliminary report [Harris, Mills and Innes, 1932].

<sup>2</sup> As reported in an earlier note [Dann *et al.* 1932] no confirmation could be found for the claim of Rygh and co-workers [Rygh and Rygh, 1932, 1; Rygh, Rygh and Laland, 1932] to have identified vitamin C with methyl $\alpha$ -narcotine or irradiated narcotine, and the criticisms there made of their technique and their deductions do not seem to be met by their later communication [Rygh and Rygh, 1932, 2].

Graded daily doses of the antiscorbutic supplement are then fed, and the recovery rates noted. A series of graded doses of orange juice (standard) is fed at the same time to another set of animals<sup>1</sup>. Negative controls are also taken and permitted to stay on the scorbutic diet, to

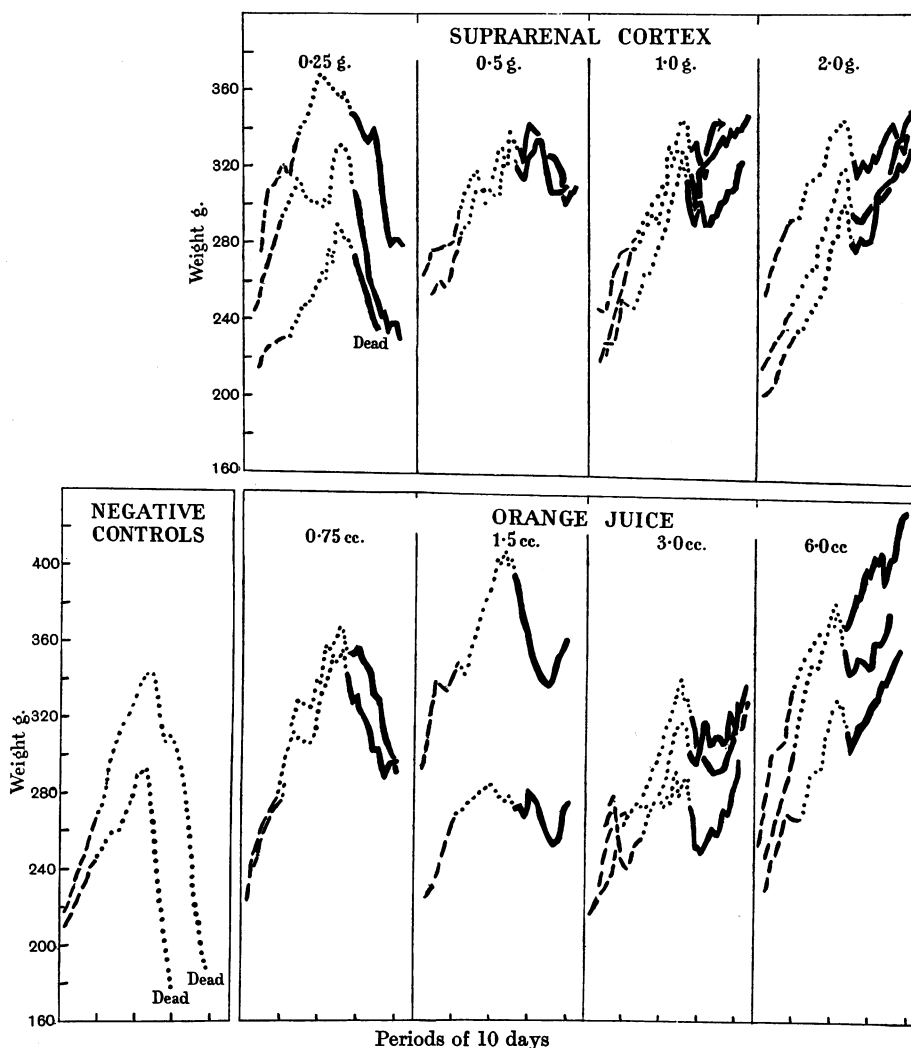


Fig. 1. Weight curves of guinea-pigs, comparing degrees of cure of scurvy with graded doses of suprarenal cortex (above) and orange juice (below).

— — — Preliminary complete synthetic diet.

..... Vitamin C-free diet.

———— Experimental period.

demonstrate that in a given batch or litter the animals are behaving regularly, and would, in fact, continue to lose weight rapidly and die from scurvy but for the addition of the antiscorbutic supplement. After a day or two, growth is resumed at full rate, provided adequacy of vitamin C has been allowed. With partial adequacy subnormal growth results (see Fig. 1), or the decline in

<sup>1</sup> Like Key and Elphick [1931], we have found orange juice preferable as standard of reference to lemon juice (the International Standard).

weight is merely stayed. Thus one can compare the doses of unknown and of standard needed to cause equal resumption in growth rate.

This method possesses a number of advantages. The demonstration of antiscorbutic activity is dramatic and unequivocal. The method is also sparing of material and is of course more rapid than the usual preventive method, since, assuming a continual supply of guinea-pigs developing scurvy, a test can safely be concluded within as short a period as 10 days. Practically the only precaution is to make no use of animals which have declined too far, and would therefore fail to respond. Animals which develop intercurrent infection or other complication to the scurvy, which would likewise prevent a normal response, should be omitted from consideration.

In the present determinations, the suprarenal cortex was tested at four levels, 0.25, 0.5, 1.0 and 2 g., and the results compared with those given by orange juice fed at the four levels of 0.75, 1.5, 3 and 6 cc. Three animals were included in each of these eight groups except that at the two lower levels of orange juice and one on suprarenal cortex only two could be spared. The individual growth curves are shown in Fig. 1. Fig. 2 gives the average gains in weight plotted against the amounts of antiscorbutic material fed.

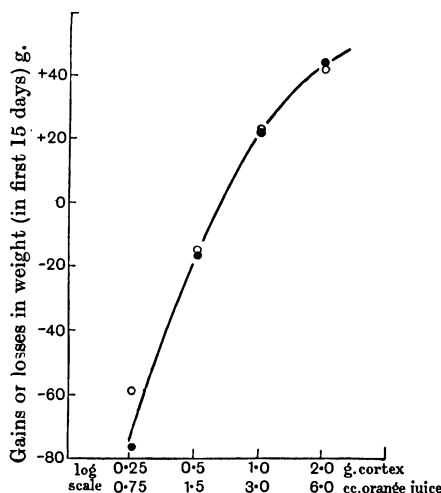


Fig. 2. Vitamin C dosage-response curve, showing average weight gain or loss with given doses of supplements.

○ Orange juice.

● Suprarenal cortex.

*Result.* It will be seen that 2.0 g. of raw suprarenal cortex corresponded very accurately with 6 cc. of orange juice, 1 g. with 3 cc., and 0.5 g. with 1.5 cc.

## 2. Tooth-structure method.

This method, based on Höjer's work, and depending on preventive action on microscopic tooth structure, has been used with satisfaction in this laboratory for the past 4 years. Normal young guinea-pigs weighing about 300 g. are given the scorbutic basal diet cited above supplemented with suitable graded daily doses of the substance to be tested. Reference animals receive graded doses of orange juice. After 14 days all the animals are killed, and cross sections of the roots of the incisors are prepared by the usual method. Comparison is made between the appearance of the teeth in the groups receiving unknown and standard (orange juice); preferably the amounts may be compared which just suffice to permit the development of normal tooth structure.

In a preliminary experiment raw suprarenal cortex was tested at levels of 0.5, 1.0 and 2.0 g., using three animals at each level. For reference orange juice was fed at levels of 1.5, 3 and 6 cc. Negative controls were given the basal diet alone.

The result of this test (Table I) showed that the minimum dose of suprarenal cortex needed for complete dental protection was approximately 1.0 g., compared with 3 cc. for orange juice.

Table I.

Material tested	Daily dose	Degree of protection on Key and Elphick's [1931] scale ("4" represents full protection)
Suprarenal cortex	0.5 g.	2, 2, 1 (av. 2)
"	1.0 g.	4, 4, 4 (av. 4)
"	2.0 g.	4, 4, 4 (av. 4)
Orange juice	1.5 cc.	3, 2 (av. 2-3)
"	3.0 cc.	4, 4 (av. 4)
"	5.0 cc.	4, 4 (av. 4)
Negative control	(No supplement)	0, 0 (av. 0)

In a confirmatory test 5 guinea-pigs were each given 0.75 g. of suprarenal cortex daily, an amount judged to be sufficient to produce a moderate degree of protection. Reference animals had 2 cc. of orange juice. The results (Table II) are in keeping with the two earlier assays.

Table II.

Material tested	Tooth sections, degree of protection in individual animals
Suprarenal cortex, 0.75 g. daily	3, 2, 2-3, 1, 2 (av. 2-3)
Orange juice, 2.0 cc. daily	2-3, 2 (av. 2-3)
Negative control (basal diet only)	0-1, 0-1 (av. 0-1)

#### *Experiments with guinea-pig suprarenals.*

Preliminary experiments on normal guinea-pig suprarenals show them to be comparable with ox suprarenals in vitamin C activity. Suprarenals from scorbutic guinea-pigs on the other hand were devoid of vitamin C (see Table III). Fuller details will be published in a later paper.

Table III.

Diet	Weight gains or losses during first week of curative test (g.)
Basal diet alone (negative control)	-72, -39
Suprarenal glands (whole) from scorbutic guinea-pigs, 0.5 g. per day	-81, -82
" " normal " "	+15, -39

### DISCUSSION.

#### *1. Vitamin C and hexuronic acid.*

The first conclusion emerging from our results is that the relative anti-scorbutic activities of suprarenal cortex and orange juice are exactly proportional to the amounts of hexuronic acid which can be isolated from these

two sources. According to Szent-Györgyi [1928], three times as much hexuronic acid can be recovered from a given weight of suprarenal cortex as from orange juice (300 mg. and 100 mg. per kg., respectively), and our experiments show that raw suprarenal cortex possesses also precisely three times the antiscorbutic activity of orange juice. This result is therefore in excellent conformity with the theory (although of course not conclusive evidence for it) that vitamin C is identical with hexuronic acid, assuming the same degree of recovery of hexuronic acid from the two sources.

Actually it is easy to show that a considerable loss of antiscorbutic material occurs during the course of isolation of the hexuronic acid in both cases. Thus, from 1 kg. of suprarenal cortex 300 mg. of hexuronic acid were isolated [Szent-Györgyi, 1928], representing according to the results of Harris, Mills and Innes not more than 300 minimum protective doses (Höjer), as compared with about 1000 doses in the original kg. of cortex itself. The loss is therefore fully two-thirds. The percentage loss is presumably identical with that from orange juice, the hexuronic acid recovered from the two sources possessing the same antiscorbutic activity (see below). While admittedly, the evidence for identifying vitamin C with hexuronic acid would have been more conclusive had the whole of the antiscorbutic activity of the original raw material been successfully concentrated in the form of the hexuronic acid separated from it, it is nevertheless too much to expect that no loss should occur when working with so unstable a material as vitamin C; and the fact that the percentage loss is the same when the same process is applied to widely different raw materials might be regarded as evidence for rather than against the theory. Furthermore the loss of vitamin seems to run parallel with that of hexuronic acid, since Szent-Györgyi [1927; 1928] estimates the amount of the latter actually present in the cortex to be two or three times that which can be recovered from it.

It is also possible to account approximately for the antiscorbutic activity of the cortex, or of orange juice, on the basis of its total hexuronic acid content, estimated chemically. For cortex, this is from 600 to 1000 mg. per kg. according to Szent-Györgyi's [1927; 1928] data; say 1000 mg. Now the minimum (Höjer) dose of hexuronic acid is from 1 to 2 mg. according to the findings of Harris, Mills and Innes; say 1 mg. Hence there are 1000 doses of 1 mg. each in the 1000 mg. of hexuronic acid in the 1 kg. of cortex. That is, the minimum dose of cortex is 1 g. This agrees with our experimental finding. But it is only by thus choosing maximum values both for the hexuronic acid content and for the activity of the hexuronic acid, that values in such good accordance with observation are reached. (This applies also in calculating the activity of other sources from their hexuronic acid contents.) The choice of the maximum value for the activity of hexuronic acid may be justifiable since it is possible that the observed value is low on account of the difficulty of preventing some inactivation. Again it seems probable that the data for hexuronic acid contents as originally given by Szent-Györgyi were under-

estimated. Thus, in good conformity with the hexuronic acid theory, we have found that the titration values of hexuronic acid and of orange juice as determined under suitable conditions by means of the reduction indicator, 2:6-dichlorophenolindophenol [Tillmans, 1930], are proportional to their relative antiscorbutic activities.

Vitamin history, however, shows the danger of assuming without the most compelling evidence that the activity of an apparently pure material is necessarily due to the main constituent and not to some unsuspected impurity. Now if one supposes the pure vitamin C to have a minimum dosage at all comparable with that of the other vitamins (D, A or B<sub>1</sub>), it follows that the activity of hexuronic acid must be due to traces of some associated impurity, amounting to no more than a small fraction of 1 %, the presence of which could easily be overlooked. It will be admitted however that there is no *a priori* justification for arguing the dosage from analogy with the other vitamins. And, while giving full weight to the above considerations, the existing evidence seems to contain nothing definitely discordant with the theory that vitamin C is identical with hexuronic acid; while, speaking more positively, it seems hard to escape the conclusion that the vitamin if not actually hexuronic acid itself is at any rate a substance possessing a close similarity to it both in its distribution and in its chemical behaviour. Whatever the final solution to the problem, this undoubtedly represents an important advance.

The evidence for the above conclusion may be summarised briefly as follows. (1) Vitamin C so far as is known has a similar distribution in nature, to hexuronic acid (*viz.* in suprarenal cortex, orange juice, cabbage<sup>1</sup>, and (?) lemon juice). (2) The specimen of hexuronic acid from suprarenals examined by us had the same degree of antiscorbutic activity as those derived from vegetable sources, tested elsewhere. (3) The widely different processes used for the isolation of (*a*) hexuronic acid by Szent-Györgyi and (*b*) vitamin C by King gave a final material possessing identical antiscorbutic activities and, apparently, chemical and physical properties. (4) Vitamin C closely follows hexuronic acid in the various precipitation and solubility reactions used in the isolation processes. (5) None of the known properties of the antiscorbutic factor is inconsistent with it being hexuronic acid. (6) As pointed out by Szent-Györgyi, Tillmans' observations on the association of antiscorbutic activity with reducing capacity are explicable on the hexuronic acid theory. Svirbely and Szent-Györgyi [1932, 2] have offered an explanation of Zilva's apparently contradictory findings in this direction. Zilva's objection [1932, 1], that he has obtained concentrates with greater activity than hexuronic acid, has been replied to by Svirbely and Szent-Györgyi [1932, 2].

<sup>1</sup> Szent-Györgyi [1928] isolated 250 mg. of hexuronic acid from 5 kg. of cabbage. Making an allowance for his acknowledged greater loss with this material the yield is in good agreement with that calculated from the antiscorbutic potency in comparison with orange juice and suprarenal. Lemon juice has the same antiscorbutic activity as orange juice and apparently a similar content of hexuronic acid although it would seem that the precise figure for the latter has not previously been determined.

The evidence of the present paper is of a remarkable degree of parallelism between hexuronic acid and antiscorbutic activity. The question can only be finally settled by further work showing whether this parallelism is invariable and complete.

## 2. *Vitamin C and the physiology of the suprarenals.*

Our results show that the suprarenal cortex possesses an astonishingly high antiscorbutic activity, hitherto quite unsuspected. The organ is considerably more potent in vitamin C than any natural source hitherto known, and certainly contains the vitamin in far greater concentration than any other animal tissue so far investigated. The liver only [Mills, 1932] may contain a greater total quantity of vitamin C, by virtue of its large bulk, but it does not compare with the suprarenal so far as localised concentration is concerned.

What is the significance of the unique collection of vitamin C in this site? The possibility suggests itself that as the liver may serve as a storehouse for vitamin C, so the suprarenal may perhaps be concerned in its elaboration or utilisation. On the other hand, it seems likely that the vitamin may be needed by the suprarenal for its functional activities—as in maintaining adrenaline-like substances in a reduced condition. One of us [Harris, 1931] has shown that the dog, unlike the guinea-pig, monkey or man, is able to synthesise its own vitamin C when none is present in the diet, and the same appears to apply to many other species, including the rat [Parsons, 1920]. Tests are in progress to determine the behaviour of the suprarenal and whether it continues to act as a source of vitamin C in such species under these conditions. We have shown that in the guinea-pig, which by contrast is unable to synthesise its own vitamin C, the suprarenal loses its antiscorbutic activity coincidentally with the onset of scurvy. The idea that the suprarenal has a specially intimate physiological connection with vitamin C gains support from the observation [McCarison, 1920] that it undergoes highly characteristic hypertrophic changes in scurvy.

## SUMMARY.

Raw suprarenal cortex (ox) is shown to be a more powerful antiscorbutic than any natural source hitherto known, possessing three times the activity of fresh orange juice (or 30 International Units per g.).

The degree of activity corresponds precisely with the value predicted on the basis of the relative yields of hexuronic acid obtainable from the two sources. (There is however a considerable loss of antiscorbutic material (the same in the two cases, *viz.* over 50 %) and of hexuronic acid during the course of the isolation.) The antiscorbutic potency of the cortex can also be accounted for on the basis of its total hexuronic acid content, as estimated chemically, provided certain assumptions are made in the calculation.

From the foregoing and other evidence it is concluded that vitamin C is either identical with hexuronic acid or is a substance possessing a close



similarity to it in its distribution and in its chemical nature, judging by its behaviour towards various solvents, precipitating agents, *etc.*

Preliminary results show that in guinea-pigs—a species which resembles man and monkeys but differs from many others, including dogs and rats, in being unable to synthesise vitamin C *in vivo* when none is provided in the food—the vitamin C activity of the suprarenal is lost with the onset of scurvy. It is supposed that vitamin C plays a special rôle in the physiology of the suprarenal. The occurrence of distinctive hypertrophic changes in the suprarenals in scurvy is in keeping with this.

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